

Cloning of the Protective Antigen Gene of *Bacillus anthracis*

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Summary

The tripartite protein toxin of *Bacillus anthracis* consists of protective antigen (PA), edema factor (EF), and lethal factor (LF). As a first step in developing a more efficacious anthrax vaccine, recombinant plasmids containing the PA gene have been isolated. A library was constructed in the *E. coli* vector pBR322 from Bam HI-generated fragments of the anthrax plasmid, pBA1. Two clones producing PA were identified by screening lysates with ELISA (enzyme-linked immunosorbent assay). Western blots revealed a full-size PA protein in the recombinant *E. coli*, and a cell elongation assay demonstrated biological activity. Both positive clones had a 6 kb insert of DNA, which mapped in the Bam HI site of the vector. The two inserts are the same except that they lie in opposite orientations with respect to the vector. Thus PA is encoded by the plasmid pBA1.

Introduction

Bacillus anthracis secretes three distinct proteins, protective antigen (PA), edema factor (EF), and lethal factor (LF), which collectively comprise the toxin of the anthrax bacillus. These proteins interact in animal test systems in combinations that produce two distinct pathological presentations. Protective antigen interacts with LF, causing death in rats (Beall et al., 1962), whereas PA in combination with EF produces localized edema in the skin of guinea pigs and rabbits (Thorne et al., 1960; Stanley et al., 1960). PA is thought to mediate the effects of EF and LF by binding to receptors of sensitive eucaryotic cells (Molnar and Altenbern, 1963). Recently, EF has been shown to be a calmodulin-dependent adenylate cyclase that dramatically elevates cyclic AMP concentrations in eucaryotic cells (Leppa, 1982).

Gladstone (1946) first demonstrated in 1948 that a soluble component from *B. anthracis* culture supernatants induces a protective immunity against subsequent challenge by live organisms. In later reports PA has been implicated as this immunogen, either by itself or in combination with another toxin component. For example, Fish et al. (1966) observed that enrichment for PA from a crude preparation improved the efficacy of the vaccine. Stanley and Smith (1963), on the other hand, reported that the combination of PA and EF was more effective than PA alone. The current vaccine licensed for human use consists of alum-precipitated supernatant material from fermenter

cultures of *B. anthracis* V770-NP1-R (Brachman et al., 1962). The growth conditions utilized for these fermenter cultures result in a supernatant that consists primarily of PA and contains barely detectable levels of EF and LF (Ristroph and Ivins, 1983; and unpublished data).

A principal goal of this research is development of an anthrax vaccine with enhanced efficacy and decreased reactogenicity. Though the cause of the reactogenicity is uncertain, it is probably caused by other *Bacillus* antigens, which may include LF and EF. PA produced from recombinant *E. coli* would be free of such antigens. The current vaccine is given in a series of six injections, followed by yearly boosters, which appear necessary to maintain protective titers of antibody. Availability of pure PA may allow larger doses to be given in each injection, so as to induce protective titers of antibody more quickly and safely.

B. anthracis contains a large plasmid (pBA1) that either regulates or encodes the production of the toxin (Mikesell et al., 1983). Plasmid-cured strains lose toxic activity (PA and/or LF) and regain it after transformation by the native plasmid DNA (Mikesell et al., 1983). By ELISA (enzyme-linked immunosorbent assay), we estimate that one cured strain produces no more than 1/2500 the amount of PA made by its wild-type parental strain (unpublished data). Such a large decrease in PA production favors the hypothesis that pBA1 codes directly for PA. However, in strains of *B. thuringiensis*, a species closely related to *B. anthracis*, the δ -endotoxin gene is apparently encoded by chromosomal DNA, plasmid DNA, or both (Schnepf and Whiteley, 1981; Klier et al., 1982; Held et al., 1982; Gonzalez, et al., 1982).

We report here the construction of a library of the plasmid DNA from a strain of *B. anthracis*. At least two of the recombinant *E. coli* clones in the library code for PA and express the protein. These *E. coli* clones offer many opportunities for clinical, diagnostic, and research applications. PA can now be isolated without contamination by other *Bacillus* proteins and indeed, even without growing *B. anthracis*. PA from the recombinant *E. coli* may eventually form the basis of an improved anthrax vaccine. Furthermore, recombinant technology will allow the examination of various domains of PA (e.g., immunogenicity, EF and LF binding, and binding to eucaryotic receptor sites).

Results

Construction of Library from pBA1

pBA1 was estimated to be 168.4 ± 7.3 kb ($X \pm SE$) as determined by electron microscopy. For purposes of cloning, a Bam HI digest with a pattern of 11 or 12 fragments was utilized (see Figure 4a). After ligation, transformation, and screening, a library of 110 colonies was collected. The DNAs (pSE 1-40) were extracted from 40 colonies and analyzed by gel electrophoresis before and after cleavage by Bam HI (data not shown). These analyses confirmed that all detectable restriction fragments generated by Bam HI digestion of pBA1 had been cloned in the *E. coli* transformants.

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Screening of the Library

Forty colonies were screened immunochemically for PA production, initially using a dot blot assay. As controls, pure PA was diluted with a lysate from *E. coli* HB101 (pBR322). Although this method, which utilizes a single antibody, resulted in high background, colonies that produced PA (or proteins that reacted with anti-PA antibody) could be distinguished from those that did not. The sensitivity for detecting PA was 5–10 ng/ml. Subsequent verification was performed by an ELISA, which employed a capture antibody and had a greatly decreased relative background (Figure 1). Two colonies (24 and 36) harboring plasmids designated pSE 24 and 36 consistently yielded positive results in both assay systems. Colony 36 often produced 2 to 4 fold more PA (10 ng/ml) than colony 24.

Further Characterization of PA-Reactive Material in Transformed *E. coli*

Although the immunochemical test showed that a peptide related to PA was being produced, these methods could not confirm whether the product was full-size and biologically active. Lysates from colonies 24, 36, and HB101 (pBR322) were analyzed by Western blotting (Figure 2). Only the lysates from colonies 24 and 36 displayed a prominent PA-reactive band, which comigrated with PA purified from *B. anthracis* (85,000 daltons). Moreover, when a lysate from colony 36, and purified PA were mixed, only one band was seen. These data show that the initial

positive response demonstrated by ELISA was due to a full-size gene product.

To determine whether a biological activity characteristic of PA was present in the recombinant *E. coli* lysates, cell elongation assays were performed. These assays were based on the demonstration (Leppa, 1982) that the combination of PA and EF causes a dramatic morphological alteration in CHO cells, due to the elevation of intracellular cAMP concentrations by the EF adenylate cyclase activity. Either PA or EF alone has no effect. CHO cells were treated with various combinations of purified PA, purified EF, and *E. coli* lysates. As anticipated, the combination of PA and EF resulted in cell elongation; cytoplasmic projections similar to dendrites could be seen at the ends of many cells. Lysates from colonies 24 and 36 could substitute for PA but not for EF (Figure 3b) in the assay. The complementation was specific, in that a lysate from *E. coli* HB101 (pBR322) in combination with pure EF did not produce the altered cell shape (Figure 3a). Moreover, titration of PA activity in the lysates agreed with previous estimates, based on the ELISA, that the lysates contain 5–10 ng PA/ml (based on original culture volume). These experiments show that the cloned gene product is biologically active.

Analysis of pSE 24 and 36 DNA

On agarose gels, pSE 24 and 36 have an identical size, estimated at 10.3 kb. To generate a restriction map, DNAs of colonies 24 and 36 were extracted and digested by

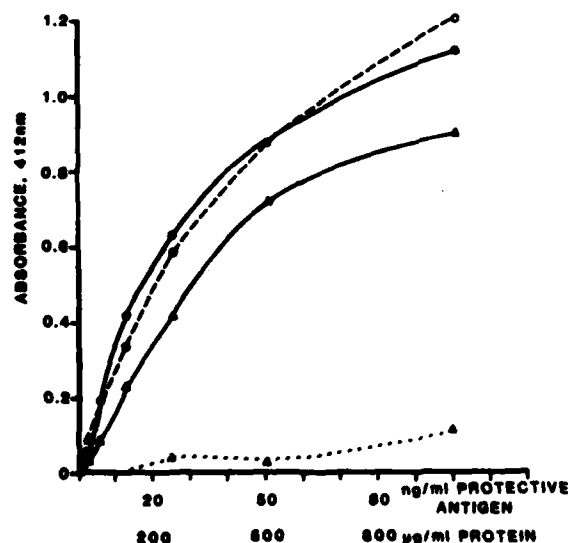


Figure 1. Titration of PA in Lysate of *E. coli* Transformants

The wells of microtiter plates previously coated with affinity-purified goat anti-PA antibody (5 µg/ml) were filled with 100 µl of purified PA (0–100 ng/ml) or with deoxycholate lysates of *E. coli* transformants (0–1000 µg/ml total protein), and with serial dilutions of these samples, made in phosphate-buffered saline, 0.05% Tween 20, 10% fetal calf serum. PA bound by the capture antibody was detected by subsequent incubations with affinity-purified rabbit anti-PA antibody, a peroxidase-labeled protein A conjugate, and the chromogenic substrate ABTS. The plate was scanned at 414 nm, and the absorbance was plotted against concentration of either PA or total protein. ○—○, purified PA; ●—●, lysate from colony 36; △—△, lysate from colony 24; ▲—▲, lysate from HB101 (pBR322).

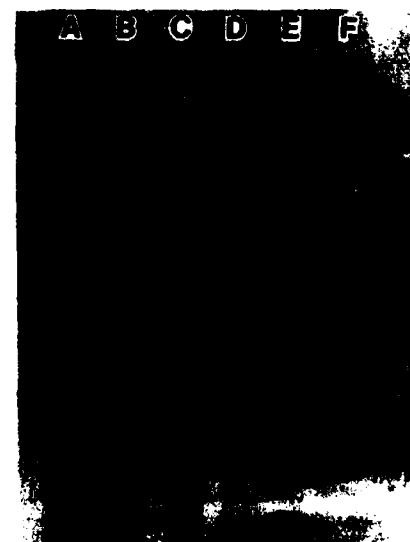


Figure 2. Western Blot of PA in Lysates from *E. coli*

Lysates from various strains of *E. coli* or PA purified from *B. anthracis* were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were transferred electrophoretically to a nitrocellulose sheet and were reacted with rabbit anti-PA antibody, which was detected with an ELISA. Lanes A and F, lysate from *E. coli* HB1010 (pBR322); lane B, lysate from colony 36; lane C, lysate from colony 24; lane D, mixture of 5 ng purified PA and lysate from *E. coli* HB101 (pBR322); lane E, mixture of 5 ng purified PA and lysate from colony 36.



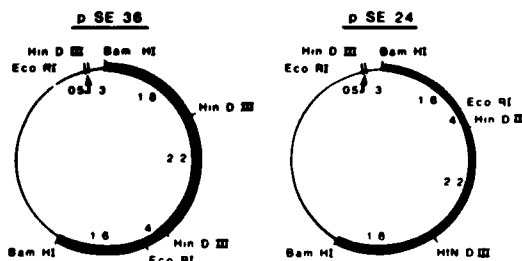
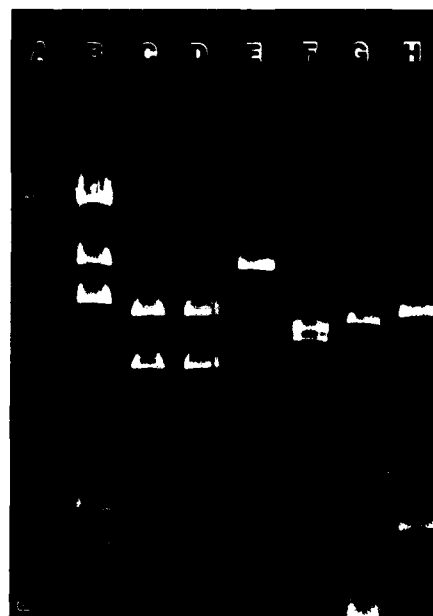
Figure 3. Elongation Response of Chinese Hamster Ovary (CHO) Cells to EF and *E. coli*-Produced PA

CHO cells were grown 24 hr in 24-well plates. Purified toxin components and dialyzed deoxycholate lysates of *E. coli* transformants were added to the indicated final concentrations. After 5 hr, the cells were photographed by phase contrast microscopy. (A) EF (200 ng/ml), and lysate from *E. coli* HB101 (pBR322) (100 μ g/ml final concentration of total protein). (B) EF (200 ng/ml) and lysate from *E. coli* colony 36 (100 μ g/ml final concentration of total protein).

several restriction enzymes (Figure 4a) known to cleave the vector at a single site. Both pSE 24 and 36 have inserts of identical size that match the 6 kb fragment of Bam HI-digested pBA1. However, the Eco RI and Hind III digests of pSE 24 and 36 differ markedly, and analysis of these differences indicates that the 6 kb inserts are in opposite orientations in the vector (Figure 4b). Since PA is produced from the 6 kb insert regardless of its orientation, this BAM HI fragment must contain both transcriptional and translational elements able to function in *E. coli*.

Discussion

A library was constructed by cloning the fragments of pBA1 into the Bam HI site of pBR322. Of 40 colonies screened immunologically for PA production, two have a positive response, and a protein was demonstrated in lysates from both colonies that comigrated with PA and reacted with anti-PA. One of the biological functions associated with PA was present specifically in the *E. coli* lysates. Thus we conclude that the PA gene from *B.*



b

Figure 4. Restriction Digests of pBA1, pSE36, and pSE24 and Restriction Maps of pSE36 and pSE24

(a) Plasmids were extracted and digested by Bam HI, Eco RI, and Hind III. Lane A, pBA1-Bam HI; lane B, λ -Hind III; lane C, pSE24-Bam HI; lane D, pSE36-Bam HI; lane E, pSE24-Eco RI; lane F, pSE36-Eco RI; lane G, pSE24-Hind III; lane H, pSE36-Hind III. (b) Restriction maps were constructed from the above data and the restriction sites published for pBR322 (Covarrubias et al., 1981).

anthracis has been cloned and that *E. coli* expresses a functional product, albeit at relatively low levels (10 ng/ml).

These results provide the first evidence that the recently discovered pBA1 directly codes for PA. Because of the complicated precedents of duplicate toxin genes in chromosomal and plasmid DNA of *B. thuringiensis* (Schnepf and Whitely, 1981; Klier et al., 1982; Held et al., 1982; Gonzales et al., 1982), it is not possible to exclude the possible existence of a PA gene in chromosomal DNA, nor is it possible to predict the location for genes encoding the other toxin components. The library of pBA1 is currently being screened for the presence of EF by techniques like these used for detecting PA.

Expression of PA by the 6 kb insert regardless of its orientation within the vector indicates the presence of a

Bacillus promoter and a ribosome-binding site. There is no obvious reason for the elevated level of PA production by colony 36 relative to colony 24, though it is possible that regulatory elements of the tetracycline-resistance gene of pBR322 have some effect. The *Bacillus* promoter must be quite strong in the homologous system as *B. anthracis* can produce up to 10 µg PA/ml culture supernatant (Ristroph and Ivins, 1983). It would be of interest to compare the strength of the PA promoter to that of other *Bacillus* promoters and to determine its usefulness for obtaining expression of other *Bacillus* genes.

A gene of 2.4–2.5 kb is required to encode a protein having the molecular weight of PA. The cloned fragment carrying the PA gene contains an additional 3.5 kb of extraneous DNA. If this extraneous DNA codes for other polypeptides, there could be complications in evaluating or producing a vaccine. Since neither EF nor LF could be detected by ELISA from the lysates of colonies 24 and 36, it is doubtful that the 6 kb insert contains an intact gene for either protein.

A number of specific genes from various species of *Bacilli* have been cloned into *E. coli*. They include genes from amylase (Yang et al., 1983; Willemot and Cornelis, 1983), sucrase (Fouet et al., 1982), penicillinase (Gray and Chang, 1981; Imanka et al., 1981), glucose dehydrogenase (Vasantha et al., 1983), δ -endotoxin (Schnepf and Whitely, 1981; Klier et al., 1982; Held et al., 1982), and for sporulation (Ollington et al., 1981; Dubnau et al., 1981; Rosenblath et al., 1981; Hirochika et al., 1981). In several cases, it has been possible to compare expression in *E. coli* with that in the donor species. Generally, expression was much less in *E. coli* for amylase, sucrase, penicillinase, and δ -endotoxin, even though the copy number was higher in *E. coli*. The one exceptional case involved glucose dehydrogenase, for which the recombinant *E. coli* had a greater specific activity. There appears to be a systematic problem for expression of *Bacilli* genes in *E. coli*, and this problem indeed affects the plans for testing the recombinant PA clones. The present level of PA in colony 36 is insufficient to conduct meaningful vaccine trials. Therefore it will be necessary to insert strong *E. coli* promoters in the recombinant DNA to boost PA production.

Experimental Procedures

Bacterial Growth and Plasmid Purification and Analysis

The strains used in this study were *B. anthracis* var. *Sterne* and a rifampin-resistant derivative selected from it by growth on solid medium containing 5 µg/ml of the drug. No detectable loss of toxin production nor curing of plasmid elements was noted in the (spontaneous) Rif^r mutant. Cultures of *B. anthracis* were grown at 37°C in a 14 l fermenter containing R medium (Ristroph and Ivins, 1983). Total cellular DNA was extracted by the method of Mura (1967), starting with at least 5 g wet weight of packed cells, and plasmid DNA was subsequently purified through two cycles of isopycnic ultracentrifugation in CsCl/ethidium bromide gradients (Maniatis et al., 1982). Dye was removed from the DNA solutions with isoamyl alcohol (Maniatis et al., 1982), and the samples were dialyzed 12 hr against TE buffer (10 mM Tris-HCl, pH 7.5; 0.1 mM EDTA). DNA was concentrated by ethanol precipitation and used either for electron microscopy (Mikesell et al., 1983) of intact plasmid or for restriction endonuclease digestion studies. The enzymes employed in this study were Bam HI, Bgl I, Eco RI, Hind III, Kpn I, Pst I, Sal I, Xba I, and T4 DNA ligase (Bethesda Research Labora-

tories). They were used according to the instructions of the manufacturer, except that a 10-fold excess of all restriction enzymes was used. Digests were analyzed by electrophoresis on 0.8% agarose gels (Maniatis et al., 1982) in TBE buffer.

Cloning of DNA

Vector (pBR322) and anthrax plasmid (pBA1) DNAs were digested to completion with Bam HI. This enzyme cleaves pBA1 into 11 or 12 fragments ranging in size from 4 to 25 kb. These fragments are two to ten times the size of the DNA required to code for PA (MW 85,000, unpublished data). The ligated DNA was introduced into *E. coli* HB101 via transformation (Bolivar and Backman, 1979), and transformants were initially selected on L agar (2% agar, 1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing 20 µg/ml ampicillin. Ampicillin-resistant transformants were then screened for the inability to grow on L agar containing 10 µg/ml tetracycline.

Screening for Production of PA

Native proteins were extracted from cell pellets of putative recombinants by treatment with lysozyme and 0.1% sodium deoxycholate (Schleif and Wensink, 1981). The clarified lysates were assayed for PA by a double antibody sandwich ELISA (Voller et al., 1979). The capture and soluble antibodies were derived from goat and rabbit, respectively. A goat was immunized with 100 µg of "Michigan antigen," (Puziss et al., 1963), in Freund's complete adjuvant. The goat was then boosted twice at intervals of 8 weeks and bled 8 weeks after the last injection. A rabbit was immunized with 50 µg of purified PA in Freund's complete adjuvant. Booster injections, which contained 50 µg of PA in Freund's incomplete adjuvant, were given seven times at average intervals of 2 weeks. The rabbit was then bled 1 month after the last injection. PA-specific antibodies were purified on columns containing PA linked to CNBr-activated Sepharose 4B (Pharmacia). The columns were prepared and operated according to the instructions of the manufacturer. A capture antibody was adsorbed to microtiter plates by incubating it in the wells at a concentration of 5 µg/ml in 0.05 M Na borate (pH 8.9). Nonspecific binding sites were saturated by overlaying with 1 mg/ml bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The microtiter plates were then washed with 0.05% Tween 20 in PBS (this washing is done after each of the three next steps). The samples and PA standards were then serially diluted in 10% fetal calf serum, 0.05% Tween 20 in PBS. The samples and standards were incubated in the plates for 2 hr at 37°C. A second (soluble) antibody to PA was added at a concentration of 1 µg/ml in 0.05% Tween 20 in PBS. Horseradish peroxidase covalently linked to Staph A protein (Surois and Pain, 1981), at a final concentration of 500 ng/ml, was allowed to react with bound rabbit globulin and the peroxidase was detected colorimetrically with 2, 2'-azino-di-(3-ethyl-benz-thiazoline sulfonic acid) (ABTS) (Sigma) as the substrate (Engvall, 1980). The microtiter plates were scanned at 414 nm by a Flow Titertek Multiskan. The assay was capable of detecting PA at a concentration of 5 ng/ml.

A dot blot assay was also used for screening cultures for PA production. The *E. coli* from 10 ml cultures were pelleted and resuspended in 500 µl of 1 mg/ml lysozyme in TES buffer (0.05 M Tris-HCl, pH 8.5; 1 mM EDTA, 15% sucrose). After 10 min at room temperature, 100 µl of 25% SDS were added and the sample was boiled for 5 min. Aliquots (5 µl) of the lysates or of purified toxin components were spotted on gridded 85 mm nitrocellulose membranes (BA 85, Schleicher and Shuell), which were then incubated for 20 min in 0.05 M Hepes (pH 7.5), 0.1 M NaCl, 3% BSA. The membranes were then washed in the previous buffer containing 0.1% BSA. Following this, the membranes were incubated with affinity-purified rabbit anti-PA antibody for either 2 hr at room temperature or 18 hr at 4°C. After incubation, the membranes were reacted with horseradish peroxidase linked to Staph A protein (500 ng/ml) and the enzyme reaction was visualized with 250 µg/ml tetramethylbenzidine solubilized in 2 mg/ml diethylenetriamine sulfonate, 5% methanol, 12.5 mM Hepes, pH 7.5 (Ron Sekura, personal communication).

Electrophoretic Analysis of Proteins

Twenty µl of *E. coli* lysates, prepared as in the dot blot assay, were subjected to SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and the proteins were electroblotted onto a nitrocellulose sheet with a transverse current (Towbin et al., 1979). Proteins that reacted with anti-PA antibody were detected by an ELISA as in the dot blot assay.

Cellular Elongation Assay

The Chinese hamster ovary (CHO) cell line and its growth conditions have already been described (Leppa, 1982). Native protein preparations from *E. coli* were dialyzed against 0.05 M HEPES (pH 7.5), 0.1 M NaCl and were incubated in 24-well plates with various combinations of PA and EF overlaid on the cell monolayer. The cell monolayer was photomicrographed under 400× magnification 3–5 hr later.

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